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Mitchell et al., Mutat. Res. 70:91-105, 1980

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Genetic and Functional Analysis of the Multiple Antibiotic Resistance (*mar*) Locus in *Escherichia coli*

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A 7.8-kbp fragment of chromosomal DNA from a region controlling multiple antibiotic resistance (Mar) in *Escherichia coli* has been sequenced. Within the fragment is a potential divergent promoter region including *marO*, which contains two pairs of direct repeats, suggesting possible operator-regulatory sites. To the left of *marO* (region I) are one or two transcriptional units with three putative open reading frames (ORFs) encoding 64, 157, and 70 amino acids. To the right (region II) is a transcriptional unit containing three putative ORFs (ORF125/144, ORF129, and ORF72). Of six independent Mar mutants, four had mutations within the ORF encoding the first putative protein (ORF125/144) downstream of *marO*, including three different single-point mutations and an IS2 insertion. One of the other mutations occurred in *marO* (20-bp duplication), and the other occurred in a site in *marO* or ORF144 (a 1-bp change). All six mutations led to increased transcription of the region II transcript. High-copy-number plasmids containing *marO* and the adjacent ORF125/144 region from a wild-type source but not from a Mar mutant reduced the antibiotic resistance of a Mar mutant to levels comparable to those of wild-type cells. High-copy-number plasmids containing wild-type *marO* alone caused an increase in resistance to tetracycline, chloramphenicol, and norfloxacin in a wild-type strain. The nature of the Mar mutations and the results of the complementation studies suggest that ORF125/144 encodes a repressor (designated MarR) which acts at *marO*. The second ORF (ORF129), designated *marA*, would encode a protein, MarA, whose sequence shows strong similarity to those of a family of positive transcriptional regulators. A Tn*5* insertion in *marA* inactivated the multiresistance phenotype of Mar mutants. The function of ORF72, designated *marB*, encoding the third putative protein in the operon, and that of other ORFs detected within the 7.8-kb fragment have not yet been determined.

Multiple antibiotic resistance specified by the bacterial chromosome rather than by plasmid-borne genes has been described infrequently (10, 14, 18, 26, 29). One such multiresistance system, designated Mar (multiple antibiotic resistance), was initially discovered by selection of *Escherichia coli* resistant to low levels of tetracycline or chloramphenicol (10). These mutants showed decreased susceptibility to the selective agent as well as to many other structurally unrelated antimicrobial agents (5, 10). Insertion of transposon Tn*5* into a site, designated *marA*, near min 34 on the *E. coli* chromosome resulted in reversal of the Mar phenotype and restoration of drug susceptibility (11). The linkage to Tn*5* was exploited in the initial cloning of *marA*::Tn*5* junctional fragments (15). These fragments were then used to isolate an intact functional *mar* region from a bacteriophage λ phasmid library made from wild-type *E. coli*. Northern (RNA blot) analysis of mRNA from wild-type and Mar mutant strains revealed increased transcription of a 1.4-kb mRNA in the Mar mutants. This transcript also showed inducibility upon growth of the cultures in tetracycline- or chloramphenicol-containing medium (15). The minimum amount of DNA from the *mar* region which would restore the complete Mar phenotype in a 39-kilobase-pair (kb) deletion mutant (which included the *mar* region) was 7.8 kb. We now report the sequence of this complementing fragment, which contains the *mar* locus and adjacent regions. The sequence contains a regulatory locus which includes several putative genes for proteins thought to be involved in the Mar phenotype and its regulation. The position and nature of six independently selected *mar* mutations have been determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general microbiological methods. The *E. coli* strains and plasmids used in this study are listed in Table 1. Plasmid pMLB1109 was generously provided by M. Berman. Unless otherwise noted, cultures were grown at 30°C in L broth, containing (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose. Tetracycline hydrochloride, chloramphenicol, ampicillin, kanamycin (all from Sigma Chemical Co., St. Louis, Mo.), and norfloxacin (Merck and Co., Rahway, N.J.) were used for selective media.

Antibiotic susceptibility testing. Antimicrobial susceptibility was compared between strains by streaking them side by side on antibiotic gradient plates (9) and incubating the plates for 40 h at 30°C.

Mar mutant selection. Mar mutants were selected on MacConkey agar (Difco Laboratories, Detroit, Mich.) containing tetracycline (3 μg/ml) or chloramphenicol (7 μg/ml) after 2 to 4 days at 30°C, as described previously (10).

DNA manipulations. Plasmid DNA isolations, transformations, restriction endonuclease digestions, and DNA probe labeling were performed as previously described (15). Single-stranded M13 DNA was isolated from growing infected strains (22).

DNA sequencing. DNAs subcloned into pUC18, M13mp18, and M13mp19 were sequenced by the chain termination

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TABLE 1. Bacterial plasmids and strains

Plasmid or strain	Relevant properties	Source or reference
Plasmids		
pUC18	Multicopy vector, Amp^r	32
pKan1	pUC18 carrying *marR1* and *marA*::Tn*5* on a 5.05-kbp insert derived from AG1025; Amp^r Kan^r	15
pHSG415	Temperature-sensitive, low-copy-number vector; Amp^r Cm^r Kan^r	16
pHHM183	pHSG415 carrying *mar*$^+$ region on a 9-kbp *Pst*I fragment in orientation 1	15
pHHM184	pHSG415 carrying *mar*$^+$ region on a 9-kbp *Pst*I fragment in orientation 2	15
pHHM191	pHHM183 *marR2* mutant, selected on tetracycline (4 μg/ml), derived in HH188	15
pHHM192	pHHM183 *marR3* mutant, selected on tetracycline (4 μg/ml), derived in HH189	15
pHHM193	pHHM183 with a mutation at bp 1447 in *marR* or *marO*, selected on tetracycline (4 μg/ml), derived in HH189	15
pHHM201	pHHM183 *marO1* mutant, selected on tetracycline (4 μg/ml), derived in HH185	15
pHHM203	pHHM184 *marR4* mutant, selected on tetracycline (4 μg/ml), derived in HH186	This study
pMarR(WT)	pUC18 carrying *marO*$^+$ and *marR*$^+$ on an 818-bp *Dra*I fragment from pHHM183	This study
pMarR(Mar)	pUC18 carrying *marO*$^+$ and *marR1* on an 850-bp *Dra*I-*Hpa*I fragment from pKan1	This study
pMLB1109	High-copy-number Amp^r β-galactosidase fusion vector	M. Berman
pSPC104	pMLB1109 carrying *marO*$^+$ on a 405-bp *Tha*I fragment from pKan1, orientation to region I	7
pSPC105	pMLB1109 carrying *marO*$^+$ on a 405-bp *Tha*I fragment from pKan1, orientation to region II	7
Strains		
AG100	*argE3 thi-1 rpsL xyl mtl supE44* Δ(*gal-uvrB*)	10
AG102	*marR1* mutant of AG100, selected on tetracycline	10
AG1025	AG102 containing *marA*::Tn*5*	11
PLK1738	*trpA his-29 ilv pro-2 arg-427 thyA deo tsx gyrA rac zdd-230*::Tn*9* Trp^r Cm^r; deletion of approximately 39 kbp from 33.6 to 34.3 min, including the *mar* locus (Δ33.6–34.3)	P. Kuempel
HH180	*supE44 hsdR endA1 pro thi zdd-230*::Tn*9* and Δ*mar* (Δ33.6–34.3) from PLK1738	15
HH188	HH180 containing pHHM183 (*mar*$^+$)	This study
HH189	HH180 containing pHHM184 (*mar*$^+$)	This study
CH164	Same as AG100 but contains *zdd-230*::Tn*9* and Δ*mar* (Δ33.6–34.3) from PLK1738	15
HH185	CH164 containing pHHM183 (*mar*$^+$)	This study
HH186	CH164 containing pHHM184 (*mar*$^+$)	This study
HH203	CH164 containing pHHM203 (*marR4*)	This study

method (27) with Sequenase (United States Biochemical, Cleveland, Ohio) according to the supplier's protocol. Reaction mixes were analyzed in 6% polyacrylamide gels containing 8 M urea. The initial primers used were M13 forward and reverse primers (Bethesda Research Laboratories, Gaithersburg, Md.) and a primer derived from the 5′ end of IS*50*L of Tn*5* (25). As sequence information was generated, additional 15-mer primers were designed and synthesized to extend the sequence in both directions. A total of 56 primers were used. The 7.8-kb DNA sequence is stored in GenBank (accession number M96235).

RNA analysis. RNA isolations and Northern (RNA blot) analyses were performed as described previously (15).

Computer analysis. The DNA sequences generated in this study were analyzed for restriction endonuclease sites, open reading frames (ORFs), and other structural features by the DM Sequence Analysis Program, Version 5.0 (Genetics PC-Software Center, Tucson, Ariz.). The DNA and protein sequences determined in these studies were also compared with other DNA and protein sequences in the GenBank (DNA) and Swiss-Prot (proteins) data bases by the FastDB method of Brutlag et al. (3).

RESULTS

DNA sequence of the *mar* region. Previous complementation studies suggested that a region of between 4.8 and 7.8 kb was necessary for obtaining Mar mutants in a strain bearing a 39-kb deletion which included the *mar* region (15). The entire 7.8-kb *Hpa*I-*Pst*I fragment from plasmid pHHM184, bearing a 9-kb *Pst*I fragment containing the chromosomal wild-type *mar* region, was sequenced from subfragments cloned into M13mp18 and M13mp19 by using universal and internally derived oligonucleotide primers. The sequence was determined to be 7,876 bp in length; bp 1 to 3400 are shown in Fig. 1. We focused on the bp 1000 to 2200 region, designated *mar* region II, which contains the site of the Tn*5* insertion which eliminates the Mar phenotype. Moreover, in previous experiments in which DNA fragments were used as probes of transcribed RNA from the *mar* region, an inducible transcript of 1.4 kb was seen to be a product of this region and showed greater transcriptional activation in Mar mutants than in the wild type (15).

Computer analysis of the *mar* region revealed a putative operon (the *marRAB* operon; see below) of at least 1.2 kb, containing three ORFs: one encoding 125 or 144 amino acids (depending on the suggested start site), and the other two encoding 129 and 72 amino acids, respectively, designated ORF125/144, ORF129, and ORF72 (Fig. 1). The ORFs are located downstream from a potential regulatory element which contains a nearly perfect *E. coli* −10 and −35 promoter consensus sequence as well as two pairs of direct repeat (DR) elements nearby. DR-1, 15 bp long with one mismatch at position 9 (TACTTGCC[T/A]GGGCAA), was located within the −10 to −35 region and its partner, DR-1′, was located just downstream from the predicted start of transcription. DR-1′ was part of an imperfect palindrome starting at bp 1423 (ATTACTTGCCAGGGCAACTAAT), and DR-1 was part of a similar shorter palindrome (Fig. 2). A second DR (DR-2 and DR-2′) of 9 bp (GCAACTAAT) flanked on both sides and partly overlapped the downstream part of DR-1′ (Fig. 2). The region containing the DRs and palindromic elements within and downstream of the pre-

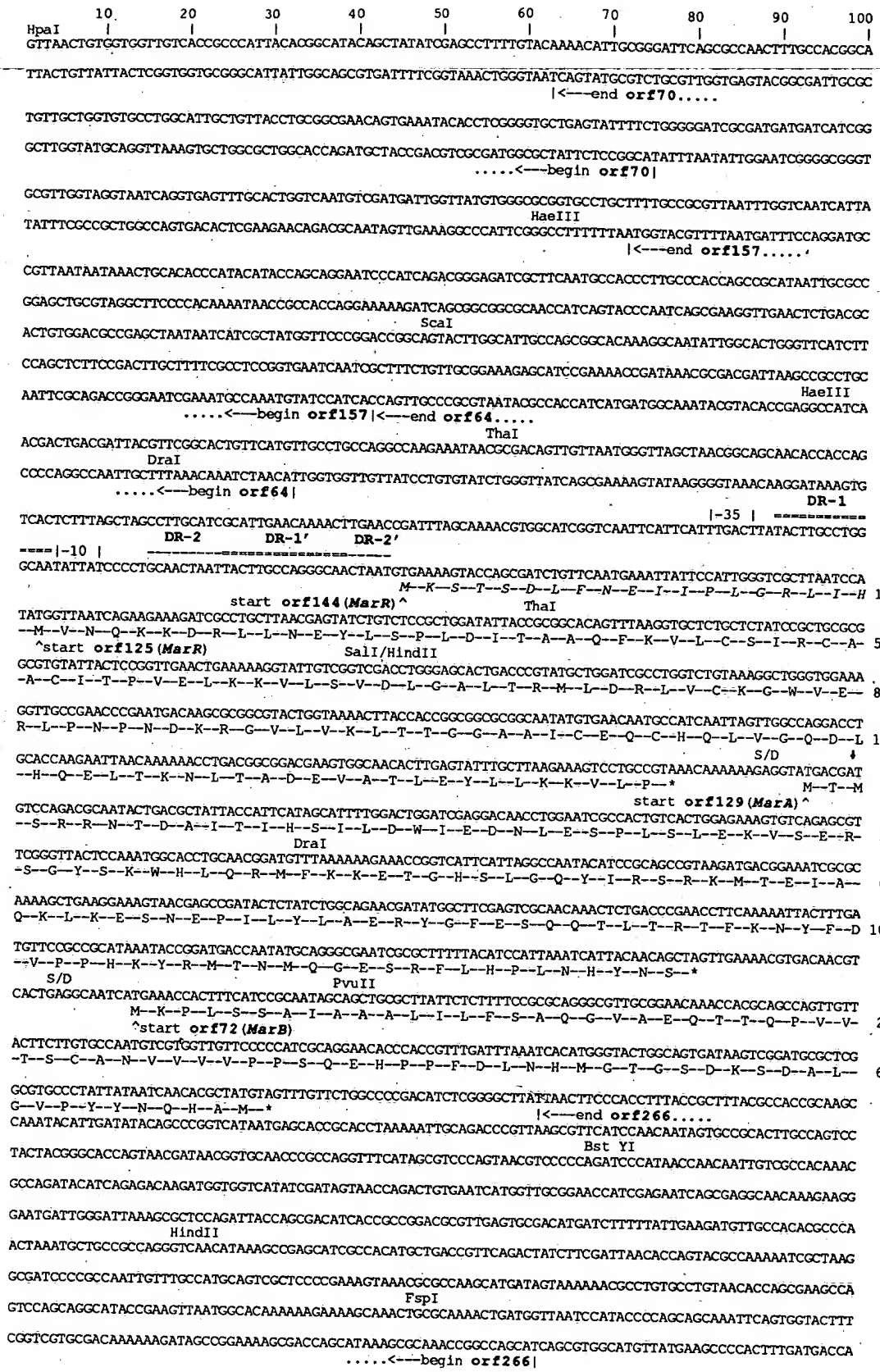

FIG. 1. Nucleotide sequence of the *mar* locus. The ORFs encoding the proteins of the *marRAB* operon are shown translated below the nucleotide sequence. Other ORFs and their directions upstream and downstream of the operon are indicated. Only those restriction endonuclease sites used during these studies are shown. Direct repeat elements DR-1 and DR-1′ (double dashed lines) and DR-2 and DR-2′ (single dashed lines) are indicated. The −10 and −35 promoter sites and Shine-Dalgarno (S/D) translational signal are also shown. The vertical arrow indicates the site of the Tn*5* insertion in AG1025.

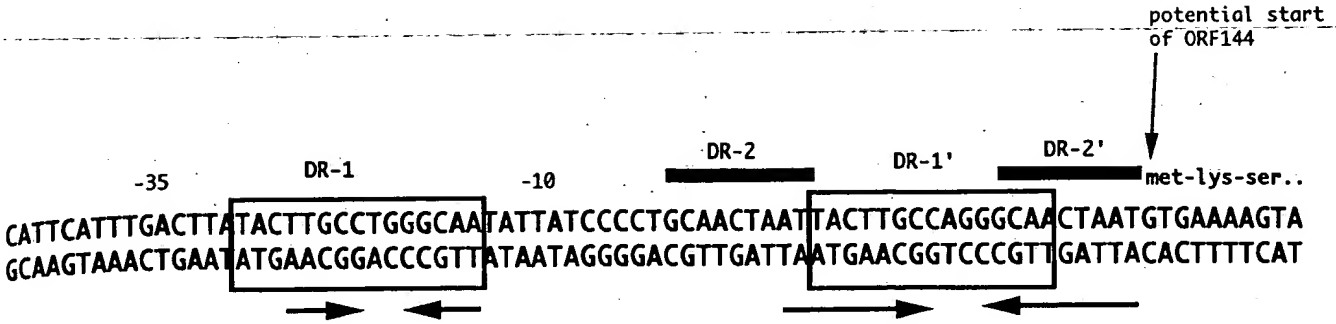

FIG. 2. Organization of the *marO* region. Direct repeats DR-1 and DR-1′ are boxed, with their internal palindromes indicated by arrows. DR-2 and DR-2′ are indicated by thick bars above the elements. The −35 and −10 promoter sequences for region II and the potential start site of ORF144 are also shown.

sumed *marRAB* promoter was considered a putative operator and was designated *marO*.

Two ORFs with putative sizes of 64 and 157 codons appear to reside on a transcriptional unit (*mar* region I) adjacent to *marO* in the direction opposite to that of the *marRAB* operon (Fig. 1). A putative promoter for region I was noted with −10 and −35 consensus sequences at positions 1350 and 1370, respectively, or 1275 and 1301, respectively. A third ORF of 70 codons may be part of the same transcriptional unit or transcribed independently. At least one, ORF157, has a hydropathy profile suggestive of a membrane protein having four transmembrane helices (data not shown). A seventh ORF of 266 codons (*mar* region III) also encodes a putative membrane protein, ends downstream of the *marRAB* operon, and would be transcribed in the opposite direction from it (Fig. 1).

As a control, we also determined the sequence from bp 1000 to 2250 of an independent clone of the wild-type *mar* region on the same 7.8-kb fragment carried in the opposite orientation in plasmid pHHM183. No differences between the wild-type *mar* sequences on pHHM183 and pHHM184 were found.

Other potential ORFs were detected in the 7.8-kb *Hpa*I-*Pst*I fragment (see Fig. 6). The entire 7.8-kb sequence of the wild-type *mar* region has been submitted to GenBank (accession number M96235).

Sequence of the *mar* locus in Mar mutants. The same cloning and sequencing strategies used for pHHM183 and pHHM184 were used to examine independent spontaneous Mar mutants (15) of pHHM183 or pHHM184 (pHHM191, pHHM192, pHHM193, pHHM201, and pHHM203), selected in a *mar* deletion strain, and pKan1 (the original partial clone), derived from AG1025. One mutation, on pHHM193, was sequenced along the entire length of the 7.8-kb *Hpa*I-*Pst*I insert; the other five were each analyzed along the *mar*-containing stretch of 1,000 bp, from nucleotides 1100 to 2100 (Fig. 1).

Comparison of the sequence of mutant pHHM193 with that of its parent pHHM183 revealed only a single base change, a G→A transversion at position 1447, which was 37 bp downstream from the putative promoter and just 3 bp downstream from the second part of the 9-bp DR element DR-2′. Depending on the start site for the first protein in the *marRAB* operon, this mutation either would lie in the codon for the first amino acid of ORF144 (converting an initial methionine to valine) or would not be within the first ORF (for ORF125). No other changes were detected within the 7,876-bp sequence.

The sequence of pKan1, a clone derived from strain AG1025, a Tn*5*-inactivated Mar mutant, was also determined because it was expected to contain the original *mar* mutation of AG102, the parental strain of AG1025 (10, 15). Indeed, the pKan1 sequence showed a single base change relative to the wild-type *mar* sequence. The mutation was a G→T change at position 1674 that would replace arginine with leucine at amino acid 58 or 77 of the product of ORF125/144, depending on the start site.

The remaining four *mar* mutations, on plasmids pHHM191, pHHM192, pHHM201, and pHHM203, were sequenced by using subcloned 1.6-kbp *Hpa*I-*Hin*dII (nucleotides 1 to 1640) and 1.3-kbp *Hin*dII (nucleotides 1640 to 3020) fragments (Fig. 1) from the region containing the putative *marRAB* operon. Each sequence revealed a unique change compared with the wild-type sequence. pHHM191 contained a T→A change at nucleotide position 1578, which replaced a valine with a glutamic acid at amino acid position 26 or 45 of

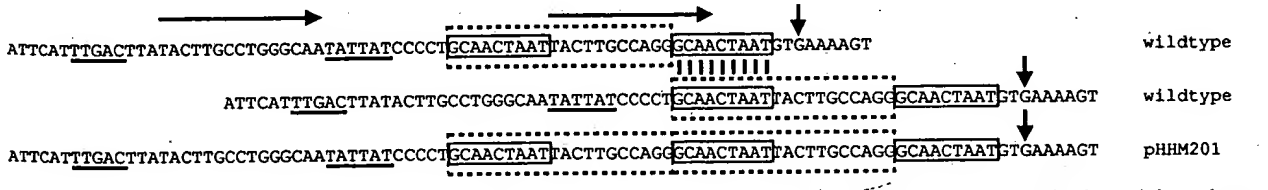

FIG. 3. Model showing the possible generation of the 20-bp duplication in *marO* of the Mar mutation on pHHM201 through homologous recombination within two 9-bp elements (DR-2; vertical bars) of two wild-type genomes. Underlined sequences, −35 and −10 consensus sequences of the putative *mar* promoter; solid-line boxes, 9-bp direct repeats; dashed-line boxes, 20-bp sequence duplicated on pHHM201; horizontal arrows, additional nearly perfect 15-bp direct repeat (DR-1; indicated only once); vertical arrows point to the position where the G→A transversion occurred in the Mar mutation on pHHM193.

ORF125/144. pHHM192 contained a G→T change at nucleotide position 1808, which converted a glutamic acid at amino acid 103 or 122 of ORF125/144 to an ocher stop codon. pHHM201 contained a 20-bp tandem duplication of a sequence (GCAACTAATTACTTGCCAGG) within the putative operator region, starting 6 bp downstream from the −10 position of the putative promoter, comprising the entire upstream part of the 9-bp DR element (GCAACTAAT) and extending into and disrupting the downstream part of the 15-bp DR element. Comparison of this mutant with the wild-type sequence revealed that a single recombination event between two wild-type DNA molecules might account for this mutation if the upstream 9-bp DR-2 element of the first DNA was paired with the downstream DR-2′ of a second DNA (Fig. 3).

Finally, pHHM203 was shown to contain an IS*2* element inserted after the second base of the codon for amino acid 73 or 91 of ORF125/144 (nucleotide position 1719); 315 bp of IS*2*-internal DNA sequence were determined from the 5′ end, and 164 bp were determined from the 3′ junction. These sequences were identical to the revised sequence of IS*2* (24). The IS*2* element was inserted in orientation II relative to the promoter for *marRAB*. As opposed to polar orientation I, which interrupts transcription of adjacent downstream genes, orientation II allows transcription from the original promoter to proceed through the entire IS*2* element and into the adjacent downstream genes (12). Consistent with the expected effect of this IS*2* insertion, Northern analysis of cells bearing pHHM203 revealed an mRNA which hybridized to the *mar*-specific probe (data not shown) that was about 1.3 kb longer than the 1.4-kb mRNA that had been found in the strain bearing the *marO* mutation of pHHM201 (15). The insertion event led to a 5-bp duplication (AATGA) at the insertion site.

In summary, all six mutants analyzed had changes within a 400-bp region. Four had a single-base-pair alteration located in ORF125/144, one had a change within the putative operator *marO*, and one had a change in either *marO* or ORF144. Five mutant plasmids (pHHM191, pHHM192, pHHM193, pHHM201, and pHHM203) caused higher expression of an RNA which hybridized to the 2.0-kb *mar*-specific probe than wild-type plasmids. Four of the five mutants had increased expression of the 1.4-kb mRNA seen in the wild-type cell, while cells bearing pHHM203, as already stated, produced increased amounts of a larger transcript, consistent with its insertional mutation (data not shown). The sixth mutation-bearing plasmid, pKan1, showed increased expression of a smaller RNA, as reported before (15).

The increased transcription in the mutants and the locations of the mutations in *marO* and *marR* suggest that ORF125/144 acts (directly or indirectly) as a repressor of transcription of region II of the *mar* locus, and this putative gene was designated *marR*. The site of repression would be the putative operator, *marO*. The downstream neighboring genes coding for ORF129 and ORF72 were designated *marA* and *marB*, respectively, of the *marRAB* operon. The mutant allele designations of the six mutant plasmids sequenced are included in Table 1.

Insertion site of Tn*5* in AG1025. In addition to the single base change in pKan1 that represented the original mutation in the chromosomal Mar mutant AG102, we also determined the insertion site of Tn*5* in AG1025 that reversed the resistance phenotype of AG102 (11). Tn*5* was located after position 1899, within the codon for the third amino acid of the *marA* product. The location was consistent with the Northern blot results reported earlier, in which a truncated but still tetracycline-inducible mRNA of 0.7 kb was found in AG1025 (as opposed to the 1.4-kb RNA in a Mar mutant [15]). These observations supported the hypothesis that *marA*, if not both *marA* and *marB* (ORF72), was necessary for expression of the Mar phenotype. The *marR* sequence (mutated in AG102) would be present in AG1025, accounting for an inducible transcript (15).

TABLE 2. Effect of wild-type and mutant MarR on antimicrobial susceptibility

Strain	Plasmid	MIC[a] (μg/ml)		
		Tetracycline	Chloramphenicol	Norfloxacin
AG100	None	2.1	7.7	0.10
	pUC18	2.1	5.8	0.08
	pMarR(WT)	1.6	4.8	0.06
	pMarR(Mar)	2.9	11.8	0.14
AG102	None	12.0	23.3	0.77
	pUC18	11.7	24.9	0.72
	pMarR(WT)	1.7	3.9	0.08
	pMarR(Mar)	9.8	24.1	0.58

[a] Antimicrobial susceptibility was determined following 40 h of incubation at 30°C on antibiotic gradient plates.

Cloning and analysis of wild-type and mutant *marR*. DNA containing *marO* and *marR* was cloned from the wild-type plasmid pHHM183 as an 818-bp *Dra*I fragment, designated pMarR(WT), while a similar but mutant region was cloned as an 850-bp *Dra*I-*Hpa*I fragment from pKan1 (bearing the original AG102 *marR1* mutation [Arg→Leu] at amino acid 58 of MarR [as ORF125]), designated pMarR(Mar). The fragments were cloned into the *Sma*I site of the high-copy cloning vector pUC18.

To test the hypothesis that MarR (the product of *marR*) acts as a repressor of the operon, we introduced both the wild-type and mutant forms of *marR* (accompanied by contiguous wild-type *marO*) into the wild-type strain AG100 and the Mar mutant AG102 (bearing the *marR1* mutation). Resistance to tetracycline, chloramphenicol, and norfloxacin was then assayed by gradient plate analysis (Table 2). Introduction of wild-type *marR* into AG100 made this strain even more susceptible to all three antimicrobial agents, whereas introduction of mutant *marR* into the wild-type cell caused a slight increase in resistance to all three agents. More dramatic, introduction of wild-type *marR* into the Mar mutant AG102 caused a large decrease in the resistance of the strain to all three agents to levels comparable to those of wild-type AG100. The mutant *marR* had no significant effect on resistance levels when introduced into AG102. These

TABLE 3. Effect of multiple copies of *marO* on antimicrobial susceptibility

Strain	Plasmid	MIC[a] (μg/ml)			
		Tetracycline	Chloramphenicol	Nalidixic acid	Norfloxacin
AG100	None	3.6	6.7	3.8	0.05
	pMLB1109	3.8	6.7	3.8	0.06
	pSPC104	7.3	9.7	5.4	0.11
AG102	None	9.8	27.2	11.1	0.33
	pMLB1109	10.0	28.9	11.8	0.33
	pSPC104	11.1	31.1	12.4	0.37

[a] See Table 2, footnote *a*.

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E.c. marA    1                     MTMSRRNTDAITIHSILD........WIEDNL.ESPLSLEKVSERSG   38
                                     II:..    .I:.::.         II:::: : II.:: I..:II
E.c. soxS    1                       MSHQK....IIQDLIA........WIDEHI.DQPLNIDVVAKKSG   32
                                   : :.I.:. . .:I. :I          :I.I:I :I :.:..I.:: .
E.c. araC  146   RYSELLAINLLEQLLLRRMEAINESLHPPMDNRVREACQYISDHLADSNFDIASVAQHVC  205
                                   :      .. . .I I  :         :II:II .. :III::.I .
P.p. xylS  180   RVQEHYAGIIASKLLEMLGSNVSREIFSKGNPSFERVVQFIEENL.KRNISLERLAELAM  238

E.c. marA   39   YSKWHLQRMFKKETGHSLGQYIRSRKMTEIAQKL...KESNEPILYLAERYGFESQQTLT   95
                 IIII.IIIII:. I :.II:III I::   I .I   :..: II: :I  .I: IIII:.
E.c. soxS   33   YSKWYLQRMFRTVTHQTLGDYIRQRRLLLAAVEL...RTTERPIFDIAMDLGYVSQQTFS   89
                 .I..:I ::I:.: I I:  :  ..::.:     I   .... II  :: . II:.I  :.
E.c. araC  206   LSPSRLSHLFRQQLGISVLSWREDQRISQAKLLL...STTRMPIATVGRNVGFDDQLYFS  262
                  I.: I .:I.I..I .  .III.II:..I . I   ...  .I  :I  III    . :.
P.p. xylS  239   MSPRSLYNLFEKHAGTTPKNYIRNRKLESIRACLNDPSANVRSITEIALDYGFLHLGRFA  298

E.c. marA   96   RTFKNYFDVPPHKYRMTNMQGESRFLHPLNHYNS            129
                 I.I:. II .I .II            I.I
E.c. soxS   90   RVFRRQFDRTPSDYR...........HRL                 107
                 I.II.: :..I .:I .  :  .  .  .I.
E.c. araC  263   RVFKKCTGASPSEFRAGCEEKVNDVAVKLS                292
                  .::. I:  I .   .  ..:.
P.p. xylS  299   ENYRSAFGELPSDTLRQCKKEVA                       321
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FIG. 4. Alignment of MarA with SoxS and AraC from *E. coli* (E.c.) and XylS from the TOL plasmid of *Pseudomonas putida* (P.p.). Percent identity (I) and percent similarity (I and :) between MarA and SoxS were 42.1 and 64.5%, respectively. Percent identity and percent similarity between MarA and AraC were 20.0 and 44.0%, respectively, and between MarA and XylS were 24.6 and 38.1%, respectively. Underlining demonstrates the amino acid identity between AraC and XylS (which showed as much amino acid identity as did MarA with either AraC or XylS). Alignment and similarity determinations were performed according to a Dayhoff table and calculated by use of the Bestfit program in the Genetics Computer Group sequence analysis software package, version 7.0, April 1991.

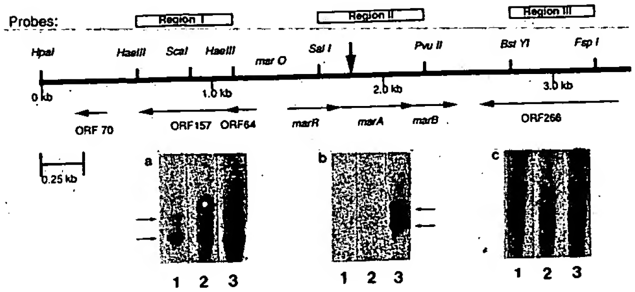

FIG. 5. Northern blot analysis with probes to regions I, II, and III. (Top) Schematic of the *mar* locus shows the DNA sequence (thick line) and ORFs (arrows). The probes for regions I, II, and III are shown (boxes). (Bottom) Northern blots of RNA from (lanes 1) HH186, (lanes 2) Mar mutant HH203, and (lanes 3) HH203 induced with tetracycline and hybridized with the region I (a), II (b), or III (c) probe. Arrows point to prominent transcripts; those in region II correspond to 1.4 and 1.0 kb (15). The white dot in the region I autoradiogram overlies an artifact on this autoradiogram.

findings suggest that MarR acts directly as a repressor of the *marRAB* operon and the Mar phenotype. Strains containing mutant forms of the protein or mutations in its putative operator region *marO* would be transcriptionally activated and show a Mar phenotype.

Cloning and analysis of *marO*. A 405-bp *Tha*I restriction endonuclease fragment bearing the whole divergent promoter region including *marO* was cloned from pKan1 into the *Sma*I site of the high-copy β-galactosidase transcriptional fusion vector pMLB1109, creating pSPC104 and pSPC105, which differed in the orientation of *marO* with respect to *lacZ* (Table 1). Expression of β-galactosidase was observed, confirming the presence of transcriptional elements in both orientations (6). pSPC104 and the host vector, pMLB1109, were introduced into AG100 and the Mar mutant AG102. Resistance to tetracycline, chloramphenicol, nalidixic acid, and norfloxacin was determined. Introduction of pSPC104 caused a 1.5- to 2-fold increase in resistance levels to all four antimicrobial agents in AG100 and a 10% increase in the already elevated resistance levels of Mar mutant AG102 (Table 3). An increase in resistance in AG100 was also seen with introduction of *marO* cloned in the opposite orientation in pSPC105, but to a lower extent than seen with pSPC104. The data suggest that in the wild-type strain, introduction of multiple copies of *marO* decreases the repression of the Mar phenotype, presumably because MarR is titrated by excess *marO*. The findings lend further support to the idea that *marO* is the site of repression by MarR.

Computer analysis of MarR, MarA, and MarB. The putative amino acid sequences of MarR, MarA, and MarB were compared with the data base of protein sequences (Swiss-Prot. version 19) by the Intelligenetics FastDB sequence alignment program (3). The analysis revealed a strong similarity between MarA and the family of positive regulators that includes several regulators involved in carbohydrate metabolism in *Escherichia coli* (AraC, RhaR, RhaS, and MelR), *Erwinia corotovora* (AraC), and *Pseudomonas putida* (XylS), virulence in *Yersinia enterocolitica* (VirF) and *E. coli* (Rns), and oxidative stress response in *E. coli* (SoxS) (1, 23, 31) (Fig. 4). MarA is substantially shorter than other members of this family (except SoxS), whose sizes range from 265 residues (Rns) to 321 residues (XylS).

A high degree of relatedness was found between MarA and SoxS. These proteins are of similar size (129 and 106 amino acids, respectively) and show homology to the carboxy-terminal portions of the other proteins in this family. The C-terminal portions of these other proteins are thought to contain DNA-binding domains, while the effector-binding site resides in the amino-terminal portions (23). An analysis of MarA by the method of Chou and Fasman (4) identified two potential helix-turn-helix DNA-binding domains located between amino acid residues 25 and 52 and between residues 79 and 92, within regions which have conserved residues with the proteins AraC and XylS (23) (Fig. 4). This obvious similarity to proteins involved in positive regulation suggests that MarA may be a transcriptional activator of genes involved in the Mar phenotype.

Considerable (50%) residue identity was also noted between MarA (amino acids 13 to 111) and the N-terminal portion (amino acids 7 to 104) of the right-origin-binding protein (RobA) of *E. coli* (28) (GenBank accession number M94042). In particular, there was striking identity in two regions of putative DNA-binding domains, 12 of 12 amino acids (GYSKWHLQRMFK) from positions 38 to 49 of MarA and 10 of 14 amino acids from positions 87 to 100 of MarA. Also noted was 43% identity of MarA with OrfR (of unknown function) in Tn*10* (27a).

MarR, the putative Mar repressor, and MarB do not show significant identity with any proteins in the Swiss-Prot data base.

Expression of regions adjacent to the *marRAB* operon. The previously described region I to the left of *marO* (Fig. 2) and ORF266 downstream of region II could each be involved in the Mar phenotype. Small DNA fragments from each of these regions were used as probes of mRNA from wild-type and Mar mutant strains (Fig. 5) to see whether any were altered in the Mar mutants.

The probe for region I consisted of a 531-bp *Hae*III fragment which is contained within the putative coding region of ORFs 64 and 157 of region I. The probe for region II was a 699-bp *Sal*I-*Pvu*II fragment including coding sequences for *marR, marA,* and *marB*. The region III probe

comprised a 479-bp *Bst*YI-*Fsp*I fragment within ORF266. Northern analysis was performed on RNA samples of wild-type (HH186) and Mar (HH203) strains with and without tetracycline induction. Region I expressed two mRNA bands in wild-type cells. These were perhaps somewhat increased in the Mar mutant but were clearly further increased in the presence of tetracycline. The region II probe confirmed the previous findings (15) that the Mar mutant produced two- to fivefold more mRNA from this region than wild-type cells. The levels of region II RNA increased further after exposure to tetracycline, two- to fivefold in the wild type and 50-fold in the Mar mutants. The potential mRNA species of region III were not detectably affected by Mar status or the presence of tetracycline.

In sum, these findings suggest that expression from region I as well as region II is altered in Mar mutants. The RNA or protein products of region I may function in the phenotypic expression of Mar in the mutated and/or induced state.

Other potential genes in the 7.8-kb sequenced fragment: identification of a sequence adjacent to the *dcp* gene. DNA sequences between 4.8 and 7.8 kb (outside of regions I, II, and III) are required to select Mar mutants in the 39-kb deletion strains (15). Therefore, the RNA or protein products of regions outside the *mar* locus are presumably needed for the Mar phenotype. Additional ORFs of more than 60 codons were recognized by the computer in the sequenced fragment (Fig. 6). Comparison of these ORFs with sequences in GenBank (release 70) revealed no known genes; however, 405 bp at the extreme right end of the fragment, up to the terminal *Pst*I site, showed complete identity with the carboxy terminus of the *dcp* gene (GenBank accession number X57947). The *dcp* gene, encoding dipeptidylcarboxypeptidase II, has recently been mapped to min 34 of the *E. coli* chromosome (2).

DISCUSSION

The *marA* site involved in the expression of chromosomal multiple antibiotic resistance, initially identified by a Tn*5* insertion, was found in the present work to lie within an operon, designated *marRAB,* with three potential protein products, MarR, MarA, and MarB. The *marA*::Tn*5* insertion has been localized to coordinate kb 1636.7 of the *E. coli* genomic map (15) at 34 min on the *E. coli* chromosome. The effects of the *marRAB* operon on expression of multiple genes suggest the existence of a *mar* regulon. One regulon member would be *micF,* whose activation in Mar mutants leads to reduction of OmpF porin, accounting for part of the multiresistance phenotype (6). Other members of the regulon could be the large number of genes encoding other membrane proteins which change in amount in Mar mutants (6). Finally, increased amounts of mRNA from transcriptional units in region I adjacent to *marO* and divergent from *marRAB* appear in Mar mutants, especially after induction of the *marRAB* operon.

The *mar* regulon may provide response to a wide variety of environmental stresses. In addition to the various classes of antimicrobial agents involved in the phenotype, the *mar* regulon appears to involve some aspects of response to oxidative stress. Greenberg et al. (13) describe a mutation selected by menadione in a locus, *soxQ,* which maps at or very near the *marRAB* operon and which is associated with changes in a number of enzymes involved in the oxidative stress response as well as conferring multiple antibiotic resistance. The Mar mutant AG102 has increased resistance to menadione and phenazine methosulfate (13). The phenotypes of the antibiotic-selected mutants (Mar) and the menadione-selected *soxQ* mutant are similar but distinguishable (13). Further analysis of the nature of the *soxQ* mutation may elucidate the reason for these differences. Other loci selected by resistance to norfloxacin (*norA* and *nfxC*) (17, 19) or ciprofloxacin (*cfxB*) (18) also map to the *mar* region and specify multidrug resistance. *cfxB* has been considered an allele of *soxQ* (13). Recently, a gene, *inaR,* associated with cellular response to internal pH change has been mapped to min 34, and *inaR* mutants show increased resistance to chloramphenicol and nalidixic acid (30). The gene may lie within the *mar* locus.

In wild-type *E. coli*, the *marRAB* operon appears to be repressed. At least two different antibiotics, tetracycline and chloramphenicol, induce *mar* operon expression at the transcriptional level. The nonantibiotic salicylate and related compounds also act as inducers (7). These findings further illustrate the adaptive and environmental stress-responsive nature of this locus. Mar mutants sequenced thus far have a mutation either in the *marO* region or in *marR,* both of which result in an apparent derepression of *marRAB* transcription.

The MarA protein is related to a family of positive transcriptional activators (23), including SoxS, XylS, AraC, and others. When present with their effectors, these proteins cause activation of a variety of different phenotypes involved in metabolism, pathogenesis, and oxidative stress in several bacterial systems. MarA may therefore act as an activator of transcription of genes (e.g., *micF* [6]) at a distance from the *marRAB* operon. Members of this family of activating proteins typically contain two functional domains, an amino-terminal portion involved in effector binding and a carboxyl-terminal portion involved in DNA binding and transcription activation (23). The MarA protein is shorter than all other known members of this protein family except SoxS and appears to consist primarily of a putative DNA-binding domain. By analogy to the larger members of this family, another protein, perhaps MarR or MarB, may fulfill the role of the effector-binding domain. Together, the two proteins might function like a single larger member of this family.

The six Mar mutants analyzed in this study contained a mutation either in *marO* or in the *marR* coding region. All six mutations lead to transcriptional activation of the operon. This finding, combined with the results of adding subclones expressing only *marO* plus a wild-type or mutant *marR* gene into wild-type or Mar mutant strains or adding multiple copies of the *marO* region alone into this background, suggests that the MarR protein is a repressor of the *marRAB* operon and acts at *marO.*

The roles of the other potential gene products identified by the sequence analysis of the region in the Mar phenotype are unknown. The putative proteins specified by region I, transcription of which is increased when *marRAB* operon expression is increased, may play functional roles in the antibiotic resistance phenotype. Their increased expression would place them in the regulon, possibly as responders to MarA, MarB, and/or MarR.

To date, studies on the mechanisms of resistance to the various classes of antimicrobial agents point to a common theme of altered transport. Enhanced active efflux of tetracycline (10), a loss of OmpF porin (6), and reduced accumulation of norfloxacin (5) in Mar mutants have been described. In addition, studies with chloramphenicol and penicillin failed to detect degradation of those compounds (10), a finding suggesting that transport changes may be a basis for resistance to these classes of agents as well. Loss of OmpF

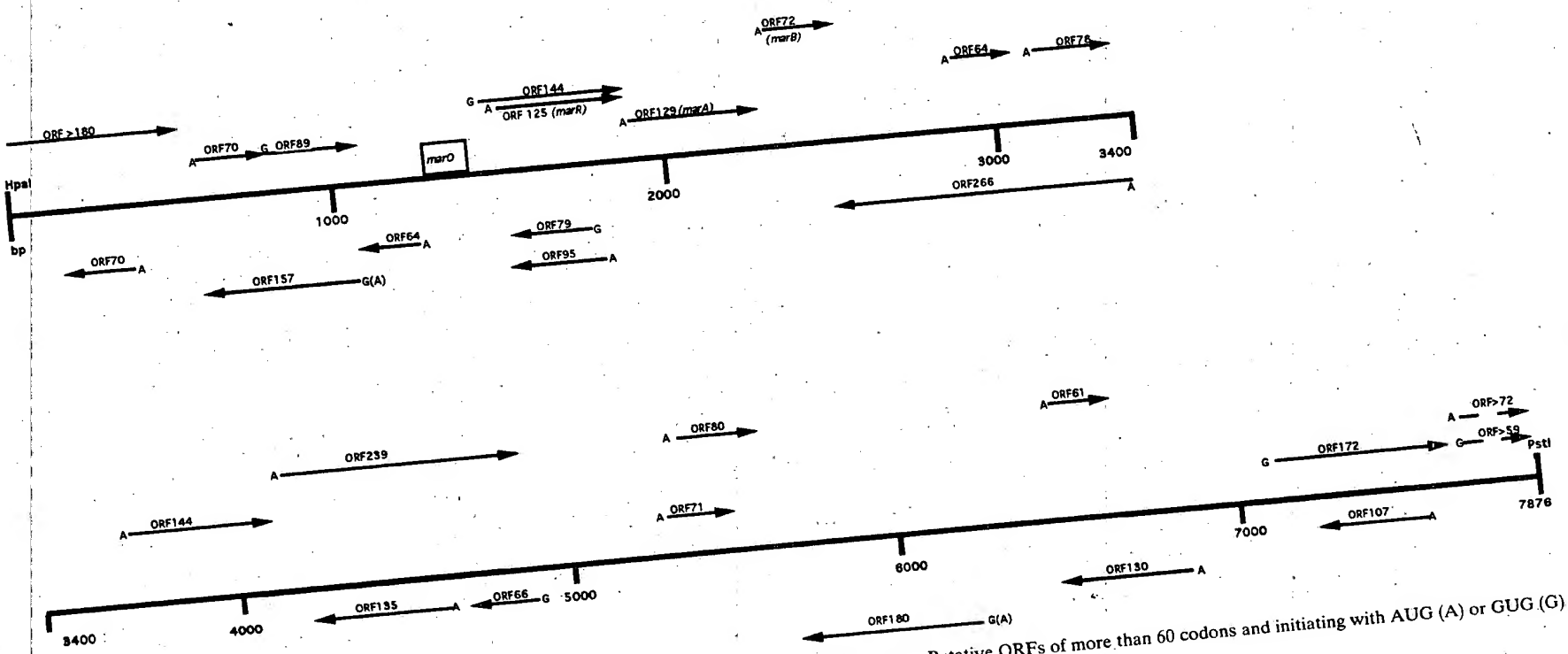

FIG. 6. Potential protein products of the sequenced 7.8-kb chromosomal region containing the *mar* locus. Putative ORFs of more than 60 codons and initiating with AUG (A) or GUG (G) are presented with their suggested transcriptional orientation, size, and start codon.

cannot account for all resistances, since OmpF mutants are less resistant than Mar mutants (6) and some of the drugs resisted do not use OmpF as a route of cell entry.

The *mar* regulon has the potential to be associated with clinical antibiotic resistance and with treatment failure. Constitutive expression of the *marRAB* operon may allow the persistence of mutants having broad-spectrum low-level resistance, with subsequent accumulation of secondary mutations leading to high-level resistance to specific agents. We know that the acquisition of high-level resistance to fluoroquinolones can occur through mutations in the *mar* operon (5). Recently, the *mar* locus has been found in other members of the family *Enterobacteriaceae*, including *Salmonella* and *Shigella* spp. (8). These findings demonstrate the potential significance of this regulated adaptive-response region to antibiotic treatment aimed at important human pathogens.

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